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Two Unlinked Genes for the Pyruvate Dehydrogenase Complex in *Aspergillus nidulans*

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The activity of the overall pyruvate dehydrogenase complex was found to be similar in extracts of *Aspergillus nidulans* after growth on either sucrose or acetate. Eight mutants lacking the activity of this complex were found among some 200 glycolytic mutants selected for their inability to grow on sucrose. The absence of pyruvate dehydrogenase complex activity was also confirmed for a mutant, g6 (*pdhA*1), isolated previously. Studies with the mutants supported the existence of two unlinked genes, *pdhA* and *pdhB*, controlling the function of the complex. In vivo and in vitro complementation between mutations at the two loci were shown by the ability of forced heterokaryons to grow on sucrose and by the restoration of overall pyruvate dehydrogenase complex activity in mixed cell-free extracts. The mutations were recessive to their wild-type alleles, and the *pdhA* and *pdhB* loci were assigned to linkage groups I and V, respectively.

The pyruvate dehydrogenase multienzyme complexes catalyze the oxidative decarboxylation of pyruvate through a series of enzyme-bound intermediates. These complexes contain three enzymatic components: pyruvate dehydrogenase (E1), dihydrolipoyl acetyltransferase (E2), and the flavoprotein, dihydrolipoyl dehydrogenase (E3). The nature of the complexes and their molecular architecture have been studied extensively by Reed and co-workers using preparations from *Escherichia coli* (18, 20) and mammalian mitochondria (17, 18). Similar pyruvate dehydrogenase complexes have been described in *Azotobacter vinelandii* (4), *Streptococcus faecalis* (19), *Saccharomyces cerevisiae* and *Kluyveromyces lactis* (17, 24), and *Neurospora crassa* (8). Regulation of the activity of the mammalian pyruvate dehydrogenase complexes by phosphorylation (inactivation) and dephosphorylation (activation) of the E1 component is mediated by a specific kinase bound to the E2 component and a loosely bound phosphatase (12, 13). Similar controls occur in *N. crassa* and *K. lactis* but not in *E. coli* or *S. faecalis* (17, 22, 25).

Genetic specification of the component enzymes of the complex has been studied extensively in *E. coli* (6, 9) and *Salmonella typhimurium* (11). Two closely linked genes (*aceE* and *aceF*) code for the E1 and E2 components, and a third closely linked gene (*lpd*) codes for the E3 component, which is also shared by the 2-oxoglutarate dehydrogenase complex.

We report here on the isolation of mutants in *Aspergillus nidulans* that lack activity of the pyruvate dehydrogenase complex, and we identify two unlinked genes which affect its function. These results offer the possibility of studying the genetic basis of the control of structure and function of the complex by introducing mutational lesions in its various components.

MATERIALS AND METHODS

Organisms and culture conditions. The mutants were isolated in a translocation-free strain R21 (*pabaA*1 *yA;* requirement for *p*-aminobenzoic acid, yellow conidia) from the Glasgow stock. A strain thought to lack pyruvate dehydrogenase complex activity, and originally called g6 (21), was supplied by A. H. Romano; it is now designated *pdhA*1 (5). The "master strain" R87 (FGSC105) (*biA*1 *AcrA*1 *wA*3 *phenA*2 *pyroA*4 *lysB*5 *sB*3 *nicB*8 *coA*1) was used in mitotic haploidization (14).

Media and culture methods used in genetic analysis and in growth of mycelium for enzyme assays were as described previously (3).

Isolation of mutants. Enrichment for glycolytic mutants was achieved by the use of a filtration technique. A suspension of washed conidia was treated (1) with *N*-methyl-*N'*- nitro-*N*-nitrosoguanidine and produced 0.5% survival. The conidia were inoculated into liquid minimal medium at a final concentration of 10^6/ml with sucrose (0.02 M) as the carbon source, and the suspension was incubated at 37°C on a gyratory shaker. Germinating conidia and mycelium were removed by aseptically filtering the suspension at intervals through cheesecloth until no further growth occurred. The viable count of surviving nongerminated conidia was determined on minimal medium with acetate (0.10 M) as the carbon source, and suitable dilutions of the suspension were

plated in this agar medium. After further incubation, the resultant colonies were replicated to sucrose minimal medium to identify the glycolytic mutants.

Genetic analysis. Conventional techniques for genetic analysis in *A. nidulans* were followed (16). Genes were allocated to linkage groups by mitotic haploidization with a "master" mapping strain (14). Diploids were inoculated on a complex medium (malt extract [Difco], 20 g; peptone [Difco], 1 g; agar [Difco], 20 g; distilled water, 1 liter) and all the nutrients required by R21 and R87 (except phenylalanine), plus sucrose (0.02 M) and acetate (0.10 M), containing either 50 mg of *p*-fluorophenylalanine or 1 mg of benlate per liter (10) to promote haploidization.

Complementation analysis was done in forced heterokaryons synthesized between *pdhA1* in the genotype *wA3 pyroA4* (white conidia, requiring pyridoxine) and the *pabaA1 yA* mutants. Heterokaryons were established on unsupplemented mineral salts medium with acetate (0.10 M) as the carbon source before transfer to the same medium but with sucrose (0.02 M) as the carbon source to test for their ability to grow on sucrose.

Enzymology. Mycelium of the R21 strain was grown in liquid minimal medium for 16 h with sucrose (0.02 M) or for 24 h with acetate (0.10 M) on a gyratory shaker at 37°C. The mutants were grown in the same way with acetate as carbon source. Mycelium was harvested and washed by filtration onto Whatman no. 1 filter paper and suspended in ice-cold 0.05 M phosphate buffer at pH 7.5. Cell-free extracts were prepared by sonication (150 W ultrasonic disintegrator, Measuring and Scientific Equipment Ltd., London; peak to peak amplitude, 12 μm) at 0°C for 20 s and centrifuged at 4°C and 18,000 × *g* for 15 min. Activity of the pyruvate dehydrogenase complex was assayed at 25°C and pH 7.5 as the pyruvate-dependant reduction of 3-acetylnicotinamide adenine dinucleotide (3-acetylNAD) (2), using a Beckman DBGT recording spectrophotometer. Protein concentrations were measured by the Folin procedure, and specific activities of the pyruvate dehydrogenase complex are expressed as micromoles of substrate transformed per hour per milligram of protein at 25°C.

In the in vitro complementation tests, equal volumes (0.05 ml) of cell-free extracts of pairs of mutants were mixed at 0°C and held for 15 min before assaying for activity of the complex. The protein concentrations in the extracts were in the range 1.5 to 4.0 mg/ml, and the activities are expressed as micromoles of substrate transformed per hour.

RESULTS AND DISCUSSION

Isolation and screening of mutants. Glycolytic mutants of *A. nidulans* are defined by their failure to grow on carbon sources, such as glucose or sucrose, while retaining the wild-type ability to utilize gluconeogenic carbon sources, such as acetate, for growth. Such mutants are readily isolated by an enrichment procedure in which a suspension of mutagenically treated conidia are incubated in liquid minimal medium with, for example, sucrose as the carbon source by using a filtration procedure to remove germinating conidia. Surviving nongrowing conidia were pour-plated in acetate minimal medium, and glycolytic mutants were identified by replicating the resultant colonies to sucrose medium.

Some 200 mutants unable to utilize sucrose were isolated, and among these a number were identified as potentially deficient in either pyruvate carboxylase or pyruvate dehydrogenase by their inability to utilize either glycerol or L-alanine (providing intracellular pyruvate) as carbon sources. These two classes of mutant can be discriminated since *pdhA1*, a strain thought to lack pyruvate dehydrogenase (21), grows on butyrate, whereas this carbon source does not support growth of pyruvate carboxylase (*pycA*) mutants (15). Although the biochemical basis of this phenotype is unknown, it was used to recognize eight more potential pyruvate dehydrogenase mutants.

Enzyme activities. Cell-free extracts of *A. nidulans* strain R21 were shown to contain overall activity of the pyruvate dehydrogenase complex by the pyruvate-dependent reduction of 3-acetylNAD. The activity of the complex was similar in extracts of sucrose- or acetate-grown mycelium (Table 1). Absence of pyruvate dehydrogenase complex activity in *pdhA1* was confirmed, and the eight new mutants were likewise found to lack this activity.

Functional relationships between *pdh* mutants. Two methods were used to establish the

TABLE 1. *Activities of the pyruvate dehydrogenase complex in A. nidulans*

Strain	Carbon source for growth	Sp act[a]
R21	Sucrose	24
R21	Acetate	38, 60, 69
pycA3[b]	Acetate	87
pdhA1	Acetate	0
106	Acetate	0
107	Acetate	0
123	Acetate	0
132	Acetate	0
139	Acetate	0
156	Acetate	0
278	Acetate	0
301	Acetate	0

[a] Micromoles of substrate transformed per hour per milligram of protein. A value of 0 is entered where no change in rate of 3-acetylNAD reduction was detected. Activities of 5% or more of wild-type extracts would have been detectable.

[b] This strain is deficient in pyruvate carboxylase (23) and was included as a relevant control.

functional relationships between the *pdh* mutants.

First, the ability of the mutant strains to complement in vivo was investigated by testing for growth on sucrose of forced heterokaryons synthesized between *pdhA*1 and each of the eight new *pdh* mutants. The mutants fell into two groups: (i) those in which the forced heterokaryons did not grow on sucrose, identifying mutants 132, 139, and 301 as having the same functional lesion as *pdhA*1, and (ii) those in which the forced heterokaryons grew vigorously on sucrose, identifying mutants 106, 107, 123, 156, and 278 as complementary to *pdhA*1 and having a different functional lesion from it.

Second, the ability of the mutant strains to complement in vitro was tested by looking for the restoration of overall pyruvate dehydrogenase complex activity when cell-free extracts of different pairs of mutants were mixed (Table 2). In each case, those mutants complementing *pdhA*1 for growth were also found to restore overall enzyme complex activity in mixed extracts, thus confirming that the two groups of mutants have different functional lesions in the enzyme complex. Moreover, those mutants not complementing *pdhA*1 for growth failed to restore activity of the enzyme complex in mixed extracts. In addition, no enzyme activity was found when one member of the group of mutants complementing *pdhA*, i.e., mutant 107, was tested in mixed extracts with the remaining members of the same group.

Taken together, these in vivo and in vitro complementation data constitute strong evidence for a second functional gene, designated *pdhB*, that affects activity of the pyruvate dehydrogenase complex in *A. nidulans*.

TABLE 2. *Restoration of activity of the pyruvate dehydrogenase complex in mixed cell-free extracts of pdh mutants of A. nidulans*[a]

Mutants tested	Activity of pyruvate dehydrogenase complex in mixed extracts with:	
	*pdhA*1	107 (*pdhB*1)
106	15.6	0[b]
107	12.0	
123	13.8	0
132	0	10.2
139	0	12.0
156	25.2	0
278	6.6	0
301	0	7.8

[a] Equal volumes of extracts from the mutant strains grown on acetate were mixed and preincubated in the cold before assay for overall pyruvate dehydrogenase complex activity (see text). Activities are expressed as micromoles of substrate transformed per hour. The activity in a similar volume of extract of strain R21 was 22 units.

[b] 0, No restoration of activity.

Moreover, since overall activity of the enzyme complex can be restored in vitro by simply mixing cell-free extracts, it is clear that the complex and its components are in a state of equilibrium such that active subunits are readily incorporated or reassembled to form an active complex.

We were not able to demonstrate conclusively which components were affected by the mutations. Attempts to assay pyruvate dehydrogenase component (E1) using the ferricyanide assay (7) were unsuccessful in the parental strain R21, presumably because the fungal complex is unable to donate electrons to ferricyanide. Mixing extracts of the *A. nidulans* mutants with extracts of one of two complementing *E. coli* mutants (A2T3 and A10) having deficiencies in the pyruvate dehydrogenase (E1, *aceE*$^-$) or acetyltransferase (E2, *aceF*$^-$) components of the *E. coli* complex produced weak overall activity. The results tentatively suggested that *pdhB* mutants are deficient in E1, and that *pdhA* mutants lack E2. However, this needs confirming when direct assays for the individual enzyme components of the *A. nidulans* complex have been developed.

Location of the *pdhA* and *pdhB* genes. The noncomplementing *pdh* mutants cannot be crossed in *A. nidulans* because the heterokaryon does not grow on hexose. However, the complementing *pdhA* and *pdhB* mutants crossed readily and segregated one-quarter wild-type (*pdh*$^+$) progeny, thus indicating absence of close linkage between the loci.

Heterozygous diploid strains were synthesized between *pdhA*1 and a master strain (R87) used to assign markers to linkage groups by mitotic haploidization (14), and between *pdhB*1 (mutant 107) and R87. In each case, the diploids grew normally upon sucrose, showing that the *pdhA*1 and *pdhB*1 mutations are recessive to their wild-type alleles.

The *pdhA* locus was assigned to linkage group I by mitotic haploidization (Table 3). Recovery of haploids carrying the mutant allele *pdhA*1 was poor, since haploidization was done on media containing sucrose and acetate, where growth of the wild-type is more vigorous than that of the mutant. This effect was even more pronounced in the case of *pdhB*1, where no haploids carrying the mutant allele were recovered (Table 3). However, allocation of the *pdhB*1 to linkage group V is unambiguous, since all the haploids were *lysB5* and none was *lysB*$^+$, showing that the configuration of chromosome pair V in the diploid must have been:

TABLE 3. *Products of haploidization of diploids R87/pdhA1 and R87/pdhB1*

Linkage group	Strain of origin of known markers[a]	Markers	No. of haploids			
			pdhA1	*pdhA*$^+$	*pdhB1*[b]	*pdhB*$^+$
I	*pdh*	*pabaA1* (*yA2*)[c]	13	0[d]	0	57
	R87	*biA1*	0	126	0	17
II	*pdh*		1	63	0	37
	R87	*AcrA1 wA3*	12	63	0	37
III	*pdh*		13	124	0	28
	R87	*phenA2*	0[d]	2[d]	0	46
IV	*pdh*		4	54	0	50
	R87	*pyroA4*	9	72	0	34
V	*pdh*		9	87	0	0
	R87	*lysB5*	4	39	0	74
VI	*pdh*		10	68	0	40
	R87	*sB3*	3	58	0	34
VII	*pdh*		7	56	0	39
	R87	*nicB8*	6	70	0	35
VIII	*pdh*		13	111	0	63
	R87	*coA1*	0[e]	15[e]	0	11[e]

[a] Genotypes are given in Materials and Methods.
[b] No *pdhB1* haploid strains were recovered. The reasons for this and the allocation of the locus to linkage group V are discussed in the text.
[c] Only haploids carrying the *wA*$^+$ allele (colored conidia) can be scored for segregation of the *yA* marker.
[d] Only haploids having the *phenA*$^+$ allele usually survive treatment with *p*-fluorophenylalanine used to cause haploidization. The diploid with *pdhB1* was haploidized with benlate (10).
[e] The *coA1* (compact colony) marker confers poor viability.

$$\begin{array}{ll} \text{R87} & \underline{lysB5\ pdhB^+} \\ 107 & \overline{lysB^+\ pdhB1} \end{array}$$

The *pdhA* locus was mapped 30 units distal to the *yA* locus by meiotic analysis.

The phenotype of the *pdh* mutants demonstrates the essential role of the pyruvate dehydrogenase complex for glycolysis in *A. nidulans*. Although the *pdh* mutants grow well upon gluconeogenic carbon sources, such as acetate, their conidiation is greatly diminished compared with R21, and this leads to poor viability of the mutant strains upon storage. As the pyruvate dehydrogenase complex is dispensible for growth on acetate, the high levels of enzyme found in extracts of *A. nidulans* grown on acetate suggest that there may be a strong metabolic control inhibiting the activity of the complex under these conditions in vivo.

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